

Long-Term Pyrene Exposure of Grass Shrimp, *Palaemonetes pugio*, Affects Molting and Reproduction of Exposed Males and Offspring of Exposed Females

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The objective of this study was to investigate the impact of long-term pyrene exposure on molting and reproduction in the model estuarine invertebrate, the grass shrimp (*Palaemonetes pugio*). Grass shrimp were exposed to measured concentrations of 5.1, 15.0, and 63.4 ppb (µg/L) pyrene for 6 weeks, during which time we determined molting and survivorship. At the end of the exposure, we immediately sacrificed some of the shrimp for biomarker (CYP1A and vitellin) analyses. The remaining shrimp were used to analyze fecundity and embryo survivorship during an additional 6 weeks after termination of pyrene exposure. Male shrimp at the highest pyrene dose (63 ppb) experienced a significant delay in molting and in time until reproduction, and showed elevated ethoxycoumarin *o*-deethylase (ECOD) activity immediately after the 6-week exposure period. In contrast, 63 ppb pyrene did not affect these parameters in female shrimp. Females produced the same number of eggs per body weight, with high egg viability (98–100%) at all exposure levels, but with decreased survival for the offspring of the 63-ppb pyrene-exposed females. In addition, vitellin levels were elevated only in females at 63 ppb pyrene after the 6-week exposure. We hypothesize that the elevated vitellin binds pyrene and keeps it biologically unavailable to adult females, resulting in maternal transfer of pyrene to the embryos. This would account for the lack of effect of pyrene exposure on ECOD activity, molting, and reproduction in the adult females, and for reduced survival of their offspring. **Key words:** cytochrome P450, molting, pyrene, reproduction, shrimp, vitellin. *Environ Health Perspect* 108:641–646 (2000). [Online 2 June 2000]

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Grass shrimp, *Palaemonetes pugio*, are a key link in the estuarine detritus food chain. Grass shrimp life history is well studied (1), and shrimp can be easily maintained in the laboratory, making them ideal and important model estuarine invertebrates. Molting and reproduction in grass shrimp are controlled by the molting hormone, ecdysone (2,3). A spike in ecdysone stimulates molting (4), and as with all steroids, ecdysone regulates gene and protein expression by interacting with its receptor, the ecdysone receptor (5). This includes the expression of ovarian egg yolk protein, vitellin (Vt). (6–8). Female grass shrimp molt, and must be mated within 7 hr of molting (1). Eggs are fertilized and extruded, then held on the pleopods of the female's abdomen until larvae are released 12–15 days later. The female molts again within a few days after spawning, and produces an additional brood. The breeding season varies with climate, but can be several months long in the estuaries of the southeastern United States. Larvae develop through a series of metamorphic molt stages and reach maturity within a few months to 1 year, depending on climate.

Estuaries often receive large amounts of anthropogenic contaminants, including polycyclic aromatic hydrocarbons (PAHs). In some areas, sediment concentrations > 4

mg/g have been found, although most highly contaminated areas are in the range of 1–2 µg/g (9–11). Water concentrations have been recorded in the nanogram-per-liter to microgram-per-liter range (10,12). In a controlled sediment-exposure study, pyrene water concentrations reached up to 18 µg/L in the filtered water from sediments containing 2.25 µg/g pyrene (12). In a separate study, grass shrimp were exposed to PAH-contaminated sediments including up to 2.1 µg/g pyrene, and water levels of pyrene reached up to 165 µg/L (10). However, in this study, water was not filtered and pyrene was most likely associated with suspended solids. Considering that pyrene levels can reach milligram-per-gram sediment levels in contaminated estuaries, it is reasonable to assume that pyrene concentrations in the field are in the microgram-per-liter range in the water column.

Biomarker induction, especially cytochrome P4501A (CYP1A) induction, has been well documented after the exposure of fish to PAHs (13). The induction of CYP1A by PAHs involves binding to the aryl-hydrocarbon receptor (AhR), which is translocated to the nucleus by the accessory protein, ARNT. The complex binds to xenobiotic response element in the promoter/enhancer region of the *CYP1A* gene,

resulting in gene transcription and ultimately CYP1A protein induction (14). The ligand that best fits into the AhR is a 10Å × 3Å planar ring (15), such as found in the PAHs and polychlorinated biphenyls.

PAHs are able to induce CYP1A-like protein activity in crustaceans as well (16–23). However, the specificity of this inducibility is slightly different from that in vertebrates. For example, 3-methylcholanthrene (3MC) does not induce ethoxyresorufin *o*-deethylase activity or benzo[*a*]pyrene hydroxylase in lobster (17), whereas in vertebrates (reptiles, birds, fish, and mammals) 3MC induces a wide variety of P450s, specifically CYP1A (24–29).

In addition to interacting with AhR, PAHs can interact with the vertebrate steroid hormone systems by acting as anti-estrogens in reporter gene assays and MCF-7 breast cancer cell proliferation assays, and as antiandrogens in whole animals (30–32). PAHs are also able to interact with the invertebrate ecdysteroid hormone system (33). Benzo[*a*]pyrene, pyrene, chrysene, and benzo[*b*]fluoranthene enhance ecdysone-dependent reporter gene expression and cell differentiation.

Several studies have been done on whole animals to investigate physiologic responses to PAH exposure in crustaceans (34–36). These studies showed that molting patterns are altered in crabs exposed to crude oil (35). Tanner crabs exposed to 0.56 mL crude oil per liter seawater autotomized (spontaneously lost) several limbs. Autotomy of limbs stimulates ecdysis (molt), and it is difficult to assess whether changes in molt are due to this physiologic response or to PAH effects. At 0.32 mL/L, molting success was increased, and crabs closest to molt were resistant to acute toxicity. Crabs exposed to PAHs during molt were less able to metabolize PAHs, presumably because PAHs are competitive substrates for some of the P450s that also

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metabolize 20-OH ecdysone to its inactive form (34,37,38). It could be argued that PAHs in molting crabs would compete with and interfere with normal molting due to interaction with the metabolism of ecdysone.

In a study using sediments from the Elizabeth River in Virginia, a PAH mixture was used that included pyrene levels up to 2 µg/g sediment and over 160 µg/L (10). At these levels, there was a slight increase in mortality from 5% in controls to 12% in exposed shrimp after 96 hr. Also, there was a slight decrease in respiration rates in shrimp exposed to the contaminated sediment.

In blue crab, PAHs are taken up primarily into the hepatopancreas, where they are metabolized and eliminated (20,39). During PAH metabolism, reactive intermediates are often produced, which lead to toxicity. Organic contaminants (dinitrochlorobenzene, hexachlorobiphenyl, and organotin) are not only distributed to the hepatopancreas for metabolism, but are also bound by Vt (the protein that serves the nutritional needs of the embryo before feeding begins). Up to 6% of the hepatopancreas burden of these contaminants was ultimately transported into the ovaries and oocytes after 6 days (40). Considering that PAHs are extremely genotoxic, this could have serious implications for the developing embryo. In fish, Vt can bind both dioxin and benzo[*a*]pyrene (41). This may be one mechanism by which organic contaminants can become biologically unavailable for metabolism to reactive intermediates, thereby mitigating toxicity to the adult animal, but promoting toxicity in their offspring.

The objective of this study was to investigate the impact of long-term exposure of pyrene on molting and reproduction in the model estuarine invertebrate, the grass shrimp. Because several PAHs, including pyrene, are able to interact with ecdysone-dependent gene transcription and cell proliferation *in vitro* (33), we hypothesized that pyrene will interfere with normal growth, reproduction, and Vt production of grass shrimp *in vivo*.

Materials and Methods

We collected grass shrimp, *Palaemonetes pugio*, near Ocean Springs, Mississippi, and held them at the Institute of Marine Science aquatic facility (Ocean Springs, MS) for 6 months before use in the exposures. Shrimp for all treatments were maintained in flow-through condition at 27°C at 15 ppt well-aerated seawater and fed *ad libitum* with brine shrimp once daily and commercial flake food once daily. Seawater was transported from the U.S. Environmental Protection Agency Environmental Research Laboratory in Gulf Breeze, Florida, adjusted

to 15 ppt salinity with nonchlorinated well water, and filtered before use. Female shrimp were separated when identified (gravid), and held separately until use.

The 6-week exposure of shrimp to pyrene was done using the methods of Walker et al. (42). We used 12 test aquaria with duplicates of three exposure levels of pyrene (10, 25, and 100 ppb) plus 2 aquaria for seawater control and 4 aquaria for solvent control [triethylene glycol (TEG)]. Four aquaria of the solvent control were used because the controlled reproductive studies required a large number of TEG (solvent)-control shrimp. Aquaria were housed within a central water bath to maintain tank temperature at 27°C. TEG concentrations in solvent controls and all treatments were kept below 0.1 mL/L seawater. We chose these levels of pyrene from preliminary static exposure 48-hr range-find assays, median lethal concentration studies from PAH sediment-exposure studies in grass shrimp, and from water concentrations reported in the literature (9,10,12). In the range-find assay, larval shrimp (< 1 month of age) were exposed to nominal concentrations of 1, 10, 100, or 1,000 ppb pyrene for 48 hr. After 48 hr, there was 60% mortality at 1 ppm and 20% mortality at 100 ppb. From the literature, a mixture of PAHs including pyrene levels of up to 165 µg/L led to 5–12% mortality after 96 hr in adult grass shrimp (10).

Before the start of the exposures, the flow-through system was allowed to operate at the selected pyrene concentrations for approximately 1 week. We measured the pyrene concentrations in the aquaria daily by HPLC [Beckman Gold System (Beckman Coulter, Inc., Fullerton, CA) with 4.6 mm × 25 cm C18 reverse-phase column eluted with 90% acetonitrile at 0.8 mL/min] with fluorometric detector [Jasco FP-920 fluorescence detector (Jasco, Inc., Easton, MD) with excitation at 235 nm and emission at 390 nm]. Pyrene concentrations (mean ± SE) were 5.1 ± 0.5, 15.0 ± 0.7, and 63.4 ± 2.5 ppb.

We exposed 50 shrimp, 25 in each of two replicate aquaria per treatment; each shrimp was housed in an individual mesh netting cage within the aquaria. This compartmentalization of test organisms precluded cannibalism, isolated males from females to avoid premature mating, and enabled us to enumerate individual molt data. We used a 16-hr light/8-hr dark photoperiod. Shrimp were checked daily for molting and survivorship. Water quality (pH, salinity, temperature, and dissolved oxygen) and pyrene levels were checked twice weekly. The average pH was 8.25 ± 0.2; salinity was 15 ± 0.4 ppt; temperature was 27.1 ± 0.3°C; and dissolved oxygen was 7.2 ± 1.8 mg/L (ppm; 100% saturation at these conditions is 7.2 ppm). There were

no differences between any of the tanks for any of these parameters.

After the 42-day exposure, a reproductive phase was initiated for an additional 6 weeks (42 days) by setting up controlled matings of treatment and control shrimp. This phase of the study was carried out in clean seawater using the following scenario: exposed males × exposed females; TEG males × exposed females; exposed males × TEG females; and seawater control males × seawater control females. Because of mortalities in the 15- and 63-ppb exposure group and 11 instances of misidentifications of sexes (e.g., female–female pairs), between 4 and 10 male–female pairs were ultimately used in the mating studies (Table 1). Pairs were checked daily for egg production, and were sacrificed after the female was gravid for a minimum of 2 days. The thorax, including hepatopancreas and gonads, was frozen at -80°C in 200 µL storage buffer (100 mM K₂HPO₄, pH 7.4, 1 mM DTT, 1 mM EDTA, 20% glycerol and aprotinin at 0.67 trypsin inhibitor units/mL). We collected, counted, and determined the viability of the eggs. A subset of 20 viable eggs/female was incubated individually in 4 mL sterile seawater/plate in 24-well polystyrene culture plates. Plates were shaken on an orbital shaker at 60 rpm at 27 ± 1°C. Percent embryo survival was determined by successful hatch by day 10 postisolation. Animals not used in the mating studies were sacrificed immediately after the 6-week exposure period, and hepatopancreas and abdomens were collected as described previously.

As a measure of CYP1A-like protein induction, we measured ethoxycoumarin *O*-deethylase (ECOD) activity in crude hepatopancreas extract. Tissues from the three collection times were used: immediately after the exposure phase; as soon as eggs were produced; and, if no eggs were produced, immediately at the end of the reproductive phase. Hepatopancreas/thorax was homogenized with a Teflon homogenizer in storage buffer and centrifuged at 14,000 rpm at 4°C in a microcentrifuge for 15 min. We used the supernatant in the ECOD assay. Protein was measured via the Bradford method (43). A 96-well plate assay was

Table 1. Numbers of pairs used in the controlled mating study.

	Male				
	Pyrene				
Female	Seawater	TEG	5 ppb	15 ppb	63 ppb
Seawater	8	—	—	—	—
TEG	—	6	8	9	5
Pyrene					
5 ppb	—	10	6	—	—
15 ppb	—	8	—	8	—
63 ppb	—	5	—	—	4

developed for the ECOD assay using the Spectramax Gemini plate reader (Molecular Devices Corp., Sunnyvale, CA) set at 380 nm excitation and 448 nm emission. Preliminary studies showed that metabolism was linear for at least 30 min at 29°C. A standard curve of hydroxycoumarin from 0 to 500 pM was used in incubation buffer (50 mM Tris, pH 7.6, 2 mM 7-ethoxycoumarin). We incubated 140 µL incubation buffer and 10 µL supernatant proteins for 5 min at 29°C. We added 3 µL NADPH (5 mM) to each well and measured fluorescence immediately for 30 min at 45 sec intervals. V_{\max} values were calculated and relative fluorescence units were converted to picomoles coumarin. Final numbers are reported as picomoles coumarin per minute per milligram protein. Each sample was measured in triplicate.

Dot-blots for Vt were done using the crude hepatopancreas/thorax homogenates that also contained gonads. The polyvinylidene difluoride (PVDF) (Millipore, Inc., Bedford, MA) membrane was wetted in methanol, rinsed in water for 5 min, and used in the BioRad dot-blot apparatus (BioRad Laboratories, Hercules, CA). Two rinses of 100 µL phosphate-buffered saline (PBS) were done under mild vacuum, and samples were applied to each well afterward. A standard curve using partially purified Vt from grass shrimp eggs (10–160 µg protein) was used to quantify Vt from samples from all phases of the study. Ten microliters of homogenates in triplicate were applied to each well of the BioRad dot-blot after heating in 2.5 µL sample buffer [0.8 M Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, and 0.05% w/v bromophenol blue] at 95°C for 5 min. We added an additional 50 µL PBS to each well, and samples were allowed to gravity filter through the unit for 2 hr at room temperature. The membrane was blocked overnight in PBS plus 3% bovine serum albumin at 4°C, rinsed 4 times with PBS plus 0.05% Tween 20 (PBST), incubated for 2 hr at room temperature with a 1:3 dilution of S-15-2 monoclonal anti-Vt antibody (44), rinsed 4 more times with PBST, and incubated 1 hr at room temperature with 1:50,000 dilution of goat-antimouse IgG coupled to horseradish peroxidase secondary antibody. Dots were visualized using the enhanced chemiluminescence kit from Amersham (Pharmacia Biotech, Inc., Piscataway, NJ), and multiple exposures of X-ray film were taken. The films were developed using a Mini-Med film processor (AFP Imaging Corporation, Elmsford, NY), and were analyzed using the BioRad gel documentation system. Western blot analysis was used to ascertain that

increased density of the dot blots was indeed due to elevated levels of Vt. To this end, a subset of homogenates (5 µg each) was subjected to separation on a 6% SDS-PAGE gel and transferred to PVDF membrane for 6 hr at 60V and blotted as described previously.

Statistical analysis was done using SYSTAT version 8.0 (SPSS, Inc., Chicago, IL) for the personal computer. Because some ECOD and embryo mortality data were non-normal, the data were square-root transformed (ECOD) or arcsine square-root transformed (percent embryo mortality) using the equation

$$x' = \sqrt{(x + 0.5)} \text{ or } p' = \arcsin(\sqrt{p})$$

(45). If outliers still remained (Durbin-Watson *t*-statistic), they were removed from the data set. Analysis of variance (ANOVA) was run on data from all assays, and if $p < 0.05$, the post hoc Tukey test was used to determine which groups were different. If data had to be transformed to meet normality requirements, ANOVA was done on transformed data. Linear regression analyses of embryo mortality were done on percentages, not on transformed data.

Results

Molting was affected only in males at the highest exposure level (Table 2). Males exposed to 63 ppb pyrene had fewer molts during the 6-week exposure period than males at other exposure levels. There was no effect of pyrene on molting in females. At 63 ppb pyrene, higher mortality occurred in both males and females (Table 2). An interesting observation was that deaths of the 63-ppb exposure group occurred at the time of, or in close proximity to, molt—shrimp were found dead while only partially or newly molted. The average day of death (\pm SE) for the males in the 63-ppb group was day 25.6

Table 2. Number of molts and percent survivorship of grass shrimp during 42 days of pyrene exposure.

Sex, treatment	No.	Survivorship (%)	Molts per shrimp ^a
Male			
Control	24	96	4.29 \pm 0.25
TEG	49	98	3.77 \pm 0.21
Pyrene			
5 ppb	23 ^b	96	3.70 \pm 0.24
15 ppb	25	100	3.60 \pm 0.22
63 ppb	14	56*	2.86 \pm 0.46*
Female			
Control	25	100	4.16 \pm 0.24
TEG	50	100	4.24 \pm 0.20
Pyrene			
5 ppb	25	100	3.96 \pm 0.25
15 ppb	24	96	3.58 \pm 0.21
63 ppb	15	60*	4.00 \pm 0.29

^aMolts per shrimp are calculated using only those shrimp surviving to day 43. ^bStarted with 24 shrimp. * $p < 0.05$.

\pm 3.5 (range 7–42); for females it was 29.5 \pm 1.7 (range 23–41) during the exposure phase of the study. Deaths occurred more frequently during the second half of the exposure study. The average number of molts before death for males was 2.4 \pm 0.5 and for females was 3.3 \pm 0.4. These animals were not used to calculate the number of molts per exposure group in Table 2. An additional two females in the 10-ppb and one female in the 63-ppb dose groups died during the following 42-day reproductive study in clean seawater.

Time until successful mating (as evidenced by gravid females) was significantly delayed in males, but not in females (Figure 1). In both groups where 63-ppb pyrene-exposed males were mated with either control females or exposed females, the time until the first brood was delayed until 24 or 14 days, respectively. Control and low-dose males were able to produce a brood after 4–7 days. By the end of the 42-day reproductive period in clean seawater, the males had recovered and there was no difference in the percent of pairs producing a brood.

Females were able to reproduce at normal levels even after exposure to the highest dose (63 ppb) pyrene (Figure 1). At all exposure levels in all groups, when females produced eggs, the number of eggs/female body weight did not differ between any exposure groups, and the percent viable eggs was not different, ranging from 98 to 100% viability (Table 3). However, there was a significant increase in embryo mortality in pyrene-exposed females at 63 ppb when mated with both control and

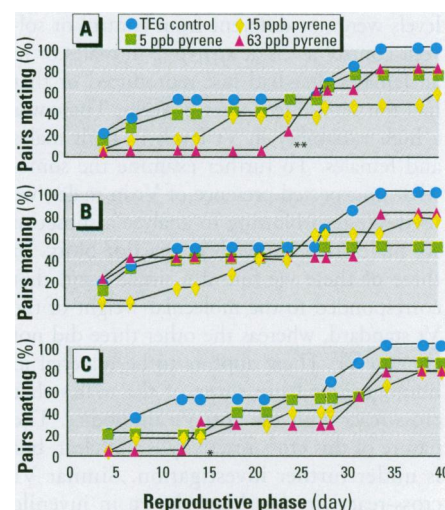


Figure 1. Number of days until production of the first brood. (A) Exposed males mated with solvent-control (TEG) females. (B) Exposed females mated with solvent-control (TEG) males. (C) Exposed males mated with exposed females. Only 63-ppb pyrene-exposed males had a significant delay in reproduction (A and C). Seawater controls and solvent-control were the same for each group, and were therefore omitted for clarity.

* $p < 0.05$; ** $p < 0.01$.

Table 3. Number of eggs per milligram female weight and percent viability.

Female	Seawater	Male			
		TEG	Pyrene		
			5 ppb	15 ppb	63 ppb
Seawater	0.53 ± 0.12 99.1%	—	—	—	—
TEG	—	0.70 ± 0.07 100%	0.66 ± 0.06 100%	0.59 ± 0.08 99.5%	0.63 ± 0.07 98.3%
Pyrene 5 ppb	—	0.62 ± 0.05 100%	0.63 ± 0.06 99.6%	—	—
15 ppb	—	0.68 ± 0.07 99.9%	—	0.71 ± 0.11 99.5%	—
63 ppb	—	0.71 ± 0.09 100%	—	—	0.74 ± 0.14 100%

exposed males (Figure 2). In addition, linear regression analysis showed a significant increase in mortality with increasing pyrene concentrations for both mating groups ($p < 0.01$ for exposed males \times exposed females, slope = 0.24; $p < 0.002$ for TEG males \times exposed females; slope = 0.66).

ECOD activity was highly variable within exposure groups (Table 4). At the end of the exposure phase, there was significant elevation of ECOD activity for males in the 63-ppb exposure group when compared to all other exposures. There was no significant induction in females after the exposure phase, and there was no significant induction of ECOD at any time during the reproductive phase of the study in either males or females (Table 4).

The percent of protein as Vt identified by dot blots was significantly elevated in females after exposure to 63 ppb pyrene for 6 weeks (Figure 3). Western blot analysis of a subset of four females confirmed these results. Vt levels were not different from control or solvent control levels at any time in males or in all females that had just reproduced or that had not reproduced after 6 weeks. There was a high variability in Vt levels in both males and females. To further examine the somewhat unexpected presence of Vt in males, we used Western blotting to analyze a subset of six males that showed Vt via dot blots. In three of these we found a single band that corresponded to the molecular weight of the Vt standard, whereas the other three did not contain Vt. There appears to be material in homogenates from some of the males that cross-react with the anti-Vt antibodies. The nature of this cross-reactivity is not clear, but is under further investigation. Similar Vt cross-reactivity has been shown in juvenile and male prawn (46). It is clear, however, that consistently low levels of apparent Vt in males do not change with pyrene exposure.

Discussion

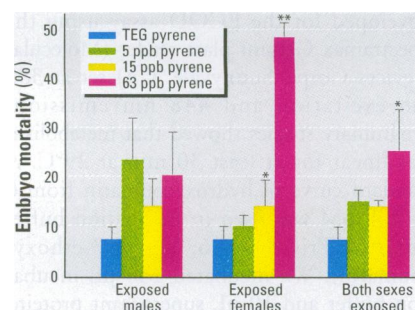
Grass shrimp are a key link in the estuarine detritus food chain. However, many of our estuaries are impacted by anthropogenic contaminants that can adversely impact this

ecologically important resource. Because grass shrimp life history is well studied (1), shrimp can be easily maintained in the laboratory, making them an ideal and important model estuarine invertebrate.

In our study, grass shrimp were exposed to 5, 15, and 63 ppb pyrene, which is similar to what has been measured in the water column in both the field and in laboratory sediment toxicity testing (10,12). Mortality was high (56% male and 60% female) at the 63-ppb dose, with shrimp dying just before or during the molt. In a study with blue crabs, animals were unable to metabolize and eliminate PAHs the closer they were to molt, resulting in a higher body burden (34). Mothershead and Hale (34) suggested that this was due to competition for substrates by P450s necessary to metabolize ecdysone for the molt. Extrapolating to this current study with a chronic pyrene exposure to shrimp, this increased PAH uptake could lead to increasingly higher body burdens of pyrene with each successive molt, eventually leading to increased mortality. In support of this hypothesis, mortality occurred primarily in the second half of the exposure study after an average of two to three molts.

The exposure of grass shrimp to pyrene resulted in significant effects on male shrimp. At the highest pyrene dose (63 ppb), males had a significantly decreased number of molts, elevated ECOD activity, and delay in mating success. Male shrimp sacrificed immediately after they mated showed that ECOD activity had returned to control levels. ECOD activity is an indicator of pyrene exposure, and it appears that once males were able to depurate enough pyrene, they were able to successfully mate.

In contrast to males, female molting, ECOD activity, and reproduction were not affected by any of these doses of pyrene. However, there is a significant induction of Vt levels at 63 ppb pyrene exposure in surviving females. Pyrene is able to increase ecdysone-dependent gene expression *in vitro* (33). Because the active ecdysteroid, 20-hydroxyecdysone (20HE), regulates protein

**Figure 2.** Embryo mortality from exposed males mated with control females, control males mated with exposed females, or both males and females exposed. There was a significant increase in embryo mortality only in the offspring from females exposed at 63 ppb.

* $p < 0.05$; ** $p < 0.001$.

Table 4. ECOD activity (pmol/min/100 μ g protein \pm SE) in grass shrimp hepatopancrei.

ECOD activity	Males, (n)	Females, (n)
Postexposure		
Control	3.39 \pm 1.86 (5)	27.51 \pm 12.66 (5)
TEG control	5.46 \pm 2.57 (5)	3.56 \pm 1.45 (5)
Pyrene		
5 ppb	13.38 \pm 4.81 (5)	10.08 \pm 5.42 (5)
15 ppb	4.35 \pm 1.78 (5)	7.22 \pm 3.98 (4)
63 ppb	42.25 \pm 8.91** (4)	13.16 \pm 4.27 (5)
Reproductive		
Control	8.00 \pm 3.13 (6)	13.63 \pm 3.58 (5)
TEG control	7.93 \pm 2.24 (22)	11.87 \pm 2.67 (21)
Pyrene		
5 ppb	12.64 \pm 3.50 (11)	17.46 \pm 3.14 (10)
15 ppb	2.33 \pm 1.28 (10)	11.58 \pm 3.26 (12)
63 ppb	9.73 \pm 3.46 (7)	13.12 \pm 2.90 (7)
Nonreproductive ^a		
Control	3.84 \pm 2.89 (4)	3.43 \pm 0.61 (4)
TEG control	8.02 \pm 2.40 (14)	5.45 \pm 1.71 (11)
Pyrene		
5 ppb	7.86 \pm 5.52 (5)	5.04 \pm 1.54 (8)
15 ppb	3.84 \pm 0.72 (9)	6.81 \pm 3.22 (7)
63 ppb	12.99 \pm 6.98 (3)	13.72 \pm 5.32 (2)

^aAnimals that did not reproduce during the reproductive phase. * $p < 0.001$ from control, TEG, and 15 ppb. ** $p < 0.03$ from 5 ppb.

expression via ecdysteroid-dependent gene expression and controls vitellogenesis (4,8), this may be the mechanism by which Vt levels are elevated in pyrene-treated shrimp. It is possible that Vt binds pyrene, resulting in reduced production of toxic reactive intermediates of PAH metabolism. This may explain the lack of adverse effects of pyrene on adult females.

Our hypothesis further predicts that Vt may serve as a vehicle for the transport of pyrene from the mother to the oocytes of embryos. Studies in blue crab and fish showed that egg yolk proteins are able to bind several classes of organic contaminants and transfer them to the oocytes (40,41). This mechanism is supported by our observed pyrene-dependent decrease in embryo survival. Interestingly, exposed females mated with solvent-control males

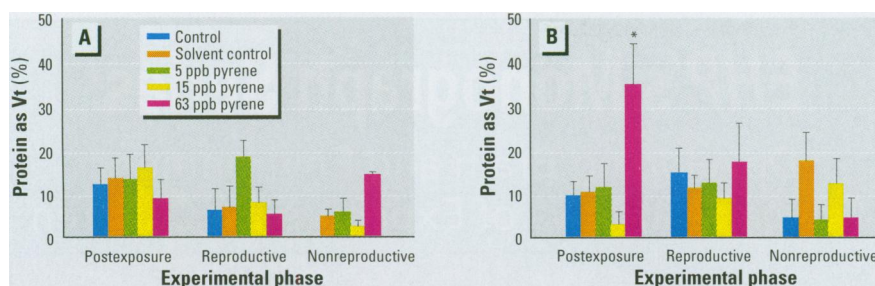


Figure 3. Vt levels in (A) male and (B) female shrimp immediately after the 6-week exposure period, 3 days after mating in clean seawater, or after 6 weeks in clean seawater if mating did not occur. Only females at 63 ppb pyrene immediately after the exposure had a significant increase in Vt levels. Vt levels in males may include cross-reacting protein; therefore, true Vt content may be overestimated.

* $p < 0.01$.

had a steeper slope in the regression analysis of embryo mortality than exposed females mated with exposed males. Successful mating of exposed males was significantly delayed, which may explain the lower slope for this group. The delay would allow for females to depurate pyrene for a longer period of time, leading to decreased pyrene in the oocytes, and less severe effects on the developing embryo, as compared to exposed females who immediately produced a brood after mating with solvent-control males.

Conclusions

This study shows that there may be a link between molting, reproduction, Vt levels, and P450 activity in shrimp. Males, but not females, are significantly delayed in molting and reproduction after exposure to environmentally relevant levels of pyrene. In the field, where exposure is continuous, this would lead to the impairment of reproductive function of male shrimp. In males, ECOD activity was significantly elevated immediately after the 6-week exposure, but in females ECOD activity was not elevated. There was no change in molting pattern or reproduction in females, but high-dose females had increased Vt levels. Our study suggests that embryo mortality would be higher in contaminated areas than in clean areas possibly because of Vt-mediated transport of PAHs from the adult to the embryos. Finally, our study shows that high pyrene concentrations can be lethal to adult shrimp after a series of molts. Taken together, these effects of PAHs may result in a reduction of the grass shrimp population, and hence decreased food availability for animals dependent on grass shrimp for prey, ultimately resulting in effects at the community and ecosystem level.

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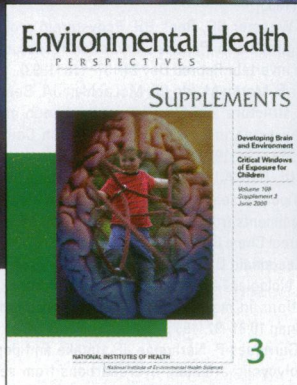
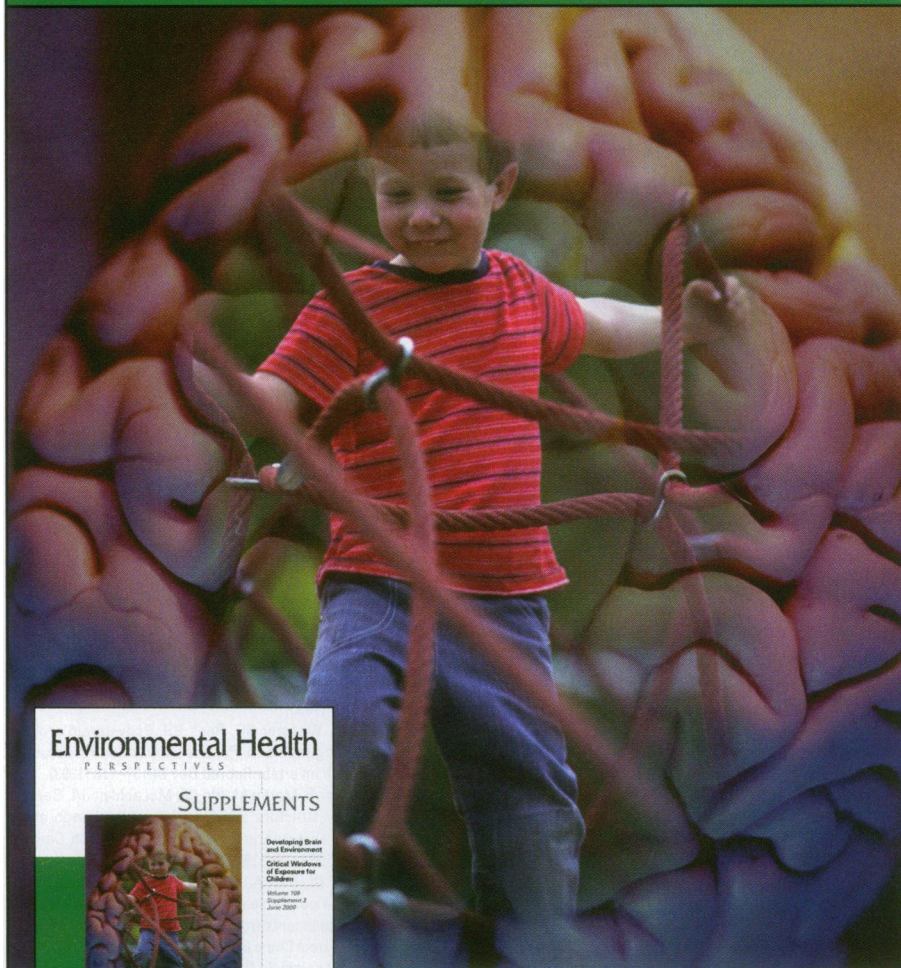
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